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Covalent flavinylation of vanillyl-alcohol oxidase is an autocatalytic process

Jianfeng Jin[1], Hortense Mazon[2,*], Robert H. H. van den Heuvel[2,†], Albert J. Heck[2], Dick B. Janssen[1] and Marco W. Fraaije[1]

1 Laboratory of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands
2 Biomolecular Mass Spectrometry and Proteomics Group, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, The Netherlands

Keywords
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Correspondence
M. W. Fraaije, Laboratory of Biochemistry, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
Fax: +31 50 3634165
Tel: +31 50 3634345
E-mail: m.w.fraaije@rug.nl

Present address
*Nancy-Université, MAEM, CNRS UMR 7567, Faculté des Sciences, Vandoeuvre, France
†NV Organon, a part of Schering-Plough Corporation, BH Oss, The Netherlands

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Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) contains a covalently 8α-histidyl bound FAD, which represents the most frequently encountered covalent flavin–protein linkage. To elucidate the mechanism by which VAO covalently incorporates the FAD cofactor, apo VAO was produced by using a riboflavin auxotrophic *Escherichia coli* strain. Incubation of apo VAO with FAD resulted in full restoration of enzyme activity. The rate of activity restoration was dependent on FAD concentration, displaying a hyperbolic relationship (K_{FAD} = 2.3 μM, $k_{activation}$ = 0.13 min^{-1}). The time-dependent increase in enzyme activity was accompanied by full covalent incorporation of FAD, as determined by SDS/PAGE and ESI-MS analysis. The results obtained show that formation of the covalent flavin–protein bond is an autocatalytic process, which proceeds via a reduced flavin intermediate. Furthermore, ESI-MS experiments revealed that, although apo VAO mainly exists as monomers and dimers, FAD binding promotes the formation of VAO dimers and octamers. Tandem ESI-MS experiments revealed that octamerization is not dependent on full covalent flavinylation.

For most reported flavoproteins, the flavin cofactor is noncovalently but tightly bound by noncovalent interactions [1]. Nevertheless, a small but significant group of flavoproteins (~ 5%) contains a covalently bound flavin. In most of these so-called covalent flavoproteins, the flavin cofactor is attached to the protein at the 8α-methyl of the isoalloxazine moiety, whereas some C6-linked flavins also have been found [2]. The most common linkage type involves coupling to a histidine residue, but proteins containing cysteinyl and tyrosyl linked flavins have also been reported. Recently, some covalent flavoproteins were even found to harbour a FAD cofactor that is tethered via two covalent linkages: a 8α-histidyl-C6-cysteinyl bound FAD [3]. The mechanism by which flavin cofactors are covalently incorporated is largely unknown, as is the rationale for covalent histidyl-flavin attachment. Previous studies have hinted at an autocatalytic process in which no helper enzymes or other additional factors are needed [2,4–6]. This is in contrast to many other

Abbreviation
VAO, vanillyl alcohol oxidase.

post-translational covalent cofactor incorporations (e.g. in the covalent tethering of the heme cofactor to *c*-type cytochromes, auxiliary enzymes facilitate the incorporation of the cofactor) [7].

Vanillyl-alcohol oxidase (VAO; EC 1.1.3.38) from *Penicillium simplicissimum* is a covalent flavoprotein containing a FAD cofactor that is bound via a common covalent linkage type: an 8α-N^3-histidyl FAD linkage [8]. VAO is highly expressed in the fungus and active with a wide variety of phenolic compounds [9,10]. Holo VAO forms homo-octamers of approximately 0.5 million Da [11–13]. The crystal structure of VAO revealed that each subunit comprises two domains [8]. The FAD-binding domain binds the ADP moiety of the FAD cofactor in an extended conformation, whereas the isoalloxazine ring of FAD is covalently attached to His422 of the cap domain. Sequence and structural alignments have revealed that VAO belongs to a family of widely distributed oxidoreductases that share a conserved FAD-binding domain [14].

To determine the functional role of the covalent flavin–protein bond in VAO, mutagenesis studies have been conducted [15,16]. Disrupting the covalent linkage by replacing the linking histidine demonstrated that the covalent bond is not needed for tight binding of FAD [15]. The crystal structure of the His422Ala mutant also revealed no structural change. Nevertheless, the noncovalent VAO mutant showed poor enzyme activity because the k_{cat} dropped by one order of magnitude. The marked decrease in catalytic activity could be attributed to a significant decrease (120 mV) in flavin redox potential. This led to the hypothesis that covalent flavinylation is crucial for effective catalysis by increasing the oxidative power of the cofactor [15]. Similar effects upon disruption of the covalent flavin–protein bond (i.e. a decrease in redox potential by approximately 100 mV and a lowered catalytic efficiency) have been observed in subsequent mutagenesis studies on other covalent flavoproteins [17–20]. For cholesterol oxidase, it was also observed that the covalent His-FAD linkage is beneficial for enzyme stability, which may hint towards an additional functional role of the covalent anchoring of the cofactor [21]. For trimethylamine dehydrogenase, the respective C6-Cys linkage prevents the enzyme from inactivation that could occur by chemical hydroxylation of the C6 position [22]. For *p*-cresol methylhydroxylase, the unus ual tyrosyl-FAD linkage has also been suggested to facilitate electron transfer from the reduced flavin to the neighbouring cytochrome subunit [5].

Although the functional role of covalent flavinylation has been elucidated for some covalent flavoproteins, the mechanistic details of how the covalent flavin–protein bond is established remain obscure. This is mainly due to difficulties in obtaining the apo form of covalent flavoproteins. Recently, the formation of a covalent Cys-FAD has been studied and described for sarcosine oxidase. To achieve this, an efficient method to produce apo sarcosine oxidase in a riboflavin auxotrophic *E. coli* strain was developed [6]. In the present study, we successfully produced apo VAO in a similar way by using another riboflavin auxotrophic *E. coli* strain. The apo VAO thus obtained was used in a detailed study of the binding of FAD and the formation of the most common covalent flavin–protein modification: an 8α-histidyl FAD linkage.

Results

Preparation and flavinylation of apo VAO

In the present study, we used *E. coli* BSV11 as expression host, which is defective in riboflavin synthesis. This riboflavin auxotrophic *E. coli* strain has been obtained by Tn5 transposon mutagenesis [23]. For production of apo VAO, cells were transformed with the expression vector pBADVAO and grown at 37 °C in LB medium, supplemented with riboflavin. Subsequently, the cells were washed and transferred to LB medium. VAO expression was induced by adding 0.2% L-arabinose to the medium and growing the cells at 17 °C. The VAO produced by this method was isolated as described previously [24]. Typically, 6 mg of VAO was obtained from 1 L of culture.

The molecular mass of the isolated VAO under denaturing conditions was determined by nanoflow ESI-MS. Accordingly, apo VAO was sprayed in 50% acetonitrile and 0.2% formic acid. This analysis revealed a molecular mass of 62 786 ± 4 Da, which is in excellent agreement with the expected mass on the basis of the VAO primary sequence, excluding the N-terminal methionine and the flavin cofactor (62 784 Da). Further evidence that the obtained VAO is mainly in the apo form came from: (a) the UV-visible spectra, which only showed some minor oxidized flavin absorbance features of the holo enzyme (Fig. 1); (b) a very low enzyme activity, which corresponded to approximately 5% of the expected activity of holo VAO; and (b) no significant flavin fluorescence upon SDS/PAGE and UV illumination (Fig. 2, lanes 1a and 2a).

Incubating 7 μM apo VAO with 100 μM FAD resulted in a strong fluorescent band (Fig. 2, lanes 1b and 2b). The fact that this fluorescent band can be observed upon SDS/PAGE provides a strong indication that the apo form can covalently incorporate the

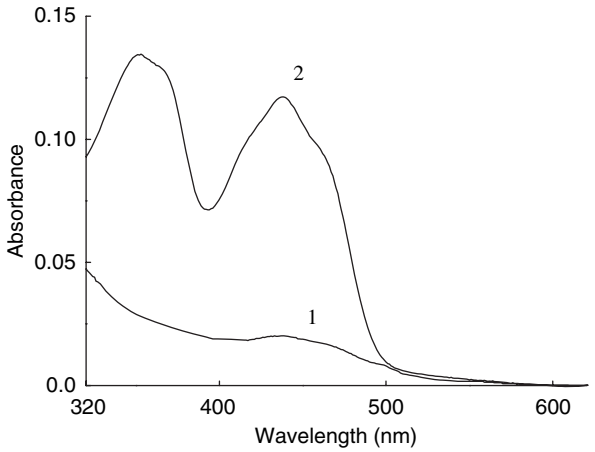


Fig. 1. Comparison of the visible absorption spectra of apo VAO (curve 1) and of the native enzyme (curve 2). Spectra were recorded in 50 mM potassium phosphate buffer (pH 7.5) at 25 °C.

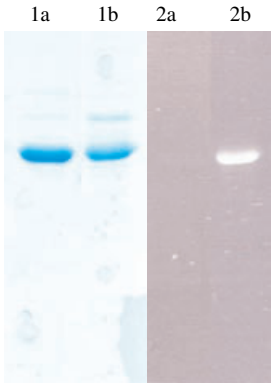


Fig. 2. Apo VAO analyzed by SDS/PAGE. Lane 1a, protein-stained purified apo VAO; lane 1b, protein-stained apo VAO pre-incubated with 100 μM FAD. Lanes 2a and 2b are identical to lanes 1a and 1b, but were analyzed for flavin fluorescence.

FAD cofactor in an autocatalytic manner. Incubation of 7 μM apo VAO with 100 μM riboflavin or FMN, in the absence or presence of ADP or AMP, did not result in any increase in enzyme activity or covalent incorporation of the respective flavin cofactor, as judged by SDS/PAGE analysis. This indicates that the complete FAD cofactor is needed for covalent incorporation.

Binding studies of apo VAO with FAD and ADP

To determine the binding affinity of apo VAO for FAD and ADP, the dissociation constants for non-covalent binding of both cofactors were determined using tryptophan fluorescence quenching (Fig. 3).

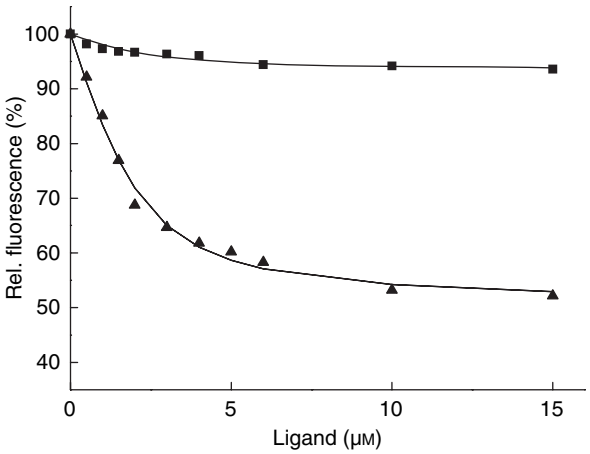


Fig. 3. Tryptophan fluorescence of apo VAO in the presence of FAD or ADP. Tryptophan fluorescence was measured upon the addition of FAD (▲) or ADP (■) to 2.0 μM apo VAO in 50 mM potassium phosphate buffer (pH 7.5). Fluorescence emission was measured at 340 nm upon excitation at 295 nm.

Titration of apo VAO with FAD or ADP resulted in a significant decrease of tryptophan fluorescence emission, as observed for apo H61T VAO [16]. When 3 μM apo VAO was incubated at 25 °C with a ten-fold excess of FAD, the fluorescence was quenched by approximately 60%, reaching a constant value within 4 min. From the titration data, a dissociation constant was determined for apo VAO: $K_{d,\ FAD} = 0.7 \pm 0.2$ μM. Binding of ADP resulted in lower fluorescence quenching, whereas a similar affinity was determined: $K_{d,\ ADP} = 1.0 \pm 0.4$ μM. The measured binding constants of wild-type apo VAO for both FAD and ADP were consistent with those of apo H61T VAO under the same conditions ($K_{d,\ FAD} = 1.8$ μM and $K_{d,\ ADP} = 2.1$ μM) [16].

Oligomerization and flavinylation of VAO

To establish the oligomerization state of apo VAO, mass spectra were recorded under nondenaturating conditions by native MS (Fig. 4A) [13,25]. Accordingly, 4 μM apo VAO was sprayed in a buffered solution (50 mM ammonium acetate, pH 6.8). One dominant ion series was observed at approximately *m/z* 5500, which corresponds to a mass of 125 577 ± 20 Da, representing dimeric apo VAO without a bound FAD. We also observed some minor satellite peaks with an increase in mass of approximately 785 Da corresponding to dimeric VAO with one bound FAD. Another minor ion series was observed at approximately *m/z* 4000 corresponding to monomeric apo VAO. Thus, the mass spectrum of apo

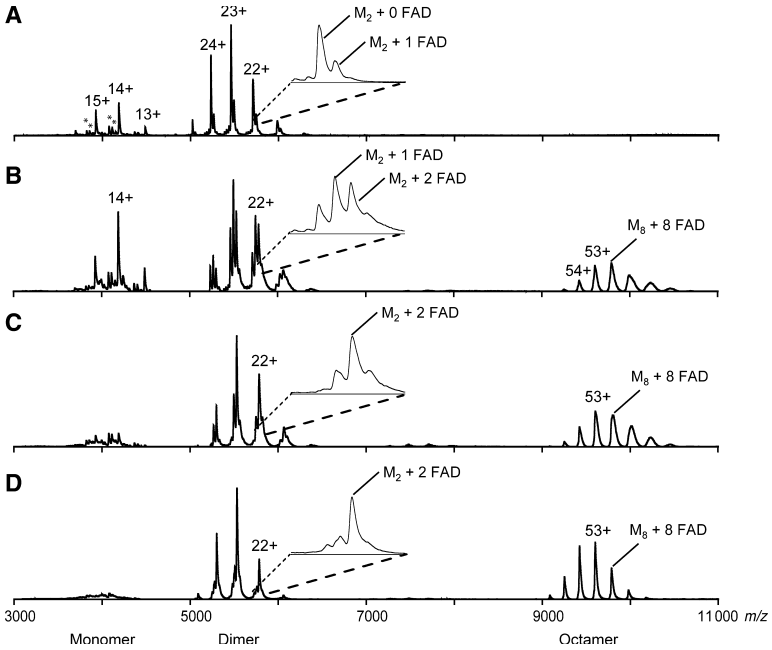


Fig. 4. Nanoflow ESI-MS of apo VAO upon addition of FAD. (A) 4 μM apo VAO in a 50 mM ammonium acetate solution (pH 6.8) and with added FAD at a ratio of protein to cofactor of 1 : 4 after incubation for (B) 1 min, (C) 24 min and (D) 162 min.

VAO (Fig. 4A) reveals that the enzyme is largely dimeric (~ 80%), whereas some monomeric species are also present.

FAD-dependent holo VAO formation was also investigated by MS under nondenaturating conditions. Flavinylation was initiated by the addition of a fourfold excess of FAD to 4 μM apo VAO in 50 mM ammonium acetate (pH 6.8). The mixtures were sprayed for MS analysis after 1, 24 and 162 min. Inspection of the spectra revealed a time-dependent formation of VAO octamers (Fig. 4B–D). After 1 min, three VAO species could be identified in the mass spectrum: monomeric (~ 20%), dimeric (~ 70%) and octameric protein (~ 10%), indicating that FAD binding is relatively fast and results in stabilization of larger oligomers. After 24 min, the monomer had decreased to approximately 5%, with the dimer being the main species (~ 80%), whereas the octamer also had increased (~ 15%). After 162 min, the dimer was still the major oligomeric form (~ 60%), the monomer had disappeared, whereas the octamer became more abundant. A close inspection of the mass spectral data of the observed dimers revealed that several dimeric species were present with varying amounts of bound FAD molecules. Indeed, three dimeric VAO species with zero, one and two FAD molecules, respectively, were found. During the reaction, the dimer without any FAD decreased with the increase of the dimer with two FAD. At 162 min, almost all dimers with zero and one FAD were converted to the dimer species with two bound FAD. We also observed that only one octameric species was present at 162 min, which contained eight FAD molecules. These data indicate that the addition of FAD to apo VAO induces oligomerization and that only a fully FAD-occupied octameric species is formed.

To determine whether the FAD molecules are covalently or noncovalently bound to the VAO octamer, tandem mass spectra were recorded under nondenaturing conditions by using nanoflow ESI-MS. The gas-phase dissociation (tandem MS) of homo-oligomers is known to expulse a monomeric subunit in its unfolded state [26,27]. This would reveal whether a FAD molecule is covalently or noncovalently bound to the monomer. Tandem mass spectra were measured after different incubation times of 4 μM apo VAO with 16 μM FAD (Fig. 5A–C). The 53^+ ion of the VAO octamer with eight bound FAD at *m/z* 9600 was isolated and the gas-phase dissociation resulted in a highly charged VAO monomer of approximately *m/z* 2000, with the concomitant formation of a lowly charged VAO heptamer of approximately *m/z* 18 000. Closer examination of the expulsed VAO monomer clearly revealed the presence of two species: VAO with zero or one covalently bound FAD. The ratio of these two species changed upon incubation time. After 4 min of incubation, we observed apo monomer and holo monomer in a ratio of approximately 1 : 1. After 12 min of incubation, the abundance of apo monomer was significantly decreased and, after 155 min of incubation, apo monomer was absent. This strongly indicates that, after 155 min, the VAO octamers were

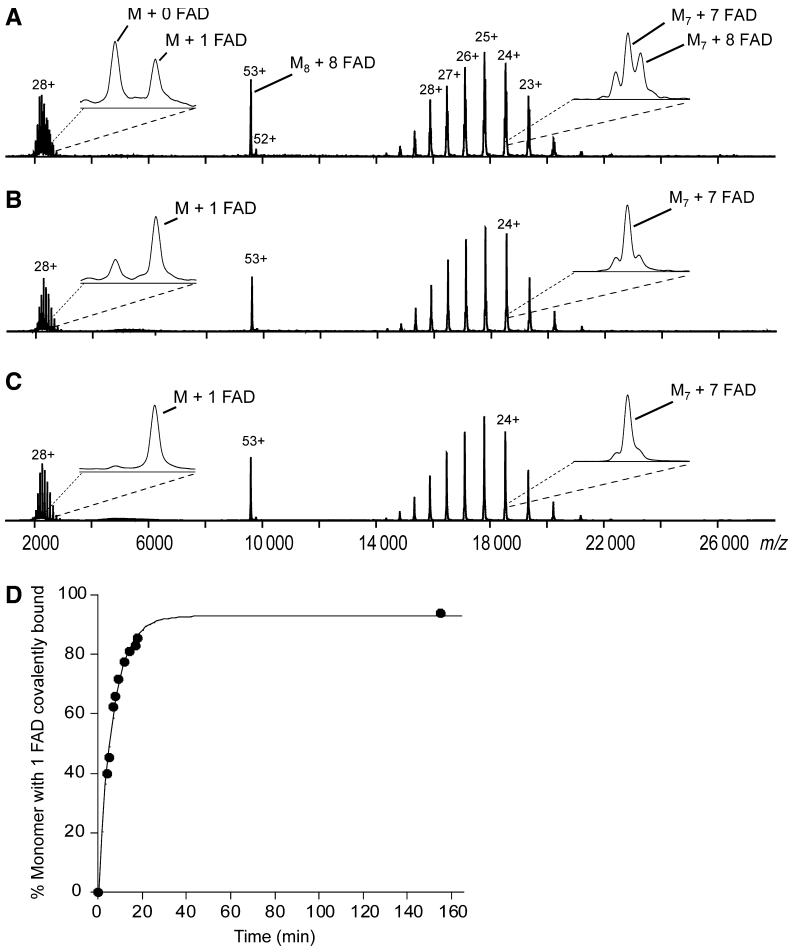


Fig. 5. Gas-phase dissociation of apo VAO upon addition of FAD. Tandem MS was performed on 4 μM apo VAO in a 50 mM ammonium acetate solution (pH 6.8) with added FAD at a ratio of protein to cofactor of 1 : 4 after incubation for (A) 4 min, (B) 12 min and (C) 155 min. (D) The formation of the covalent FAD–VAO bond over time during the reconstitution is shown as analyzed by tandem MS. Fitting the data using a single-exponential equation yielded a rate of 0.12 s^{-1}.

fully saturated with covalently bound FAD. The tandem MS data revealed a clear time-dependent process of covalent binding of FAD to VAO. Kinetic analysis of the tandem mass spectral data yielded an observed rate of 0.12 min^{-1} for covalent FAD incorporation (Fig. 5D).

The data reported above fit well with the data concerning reactivation of VAO in the presence of FAD (Fig. 6). When 1.1 μM apo VAO was incubated with 500 μM FAD in 50 mM phosphate buffer (pH 7.5) at 25 °C, the activity of VAO gradually increased over time. Immediately upon mixing the enzyme with FAD, a significant increase in activity was observed (Fig. 6A). In a subsequent relatively slow process, the activity increased even further and reached a maximum at 30 min. The final activity obtained matched well the expected value for native VAO. The covalent incorporation of FAD was followed over time by monitoring the fluorescence intensity upon SDS/PAGE (Fig. 6A, inset). The fluorescence intensity of the protein bands only reached a maximum fluorescence after 40 min, indicative of a relatively slow covalent flavinylation process. The observed rate of covalent incorporation from tandem mass spectra is similar to that measured by reactivation and SDS/PAGE analysis (Fig. 5D). These data show that the initial noncovalent FAD binding induces oligomerization of VAO and that only after a subsequent relatively slow process is the FAD cofactor covalently tethered to the protein via an autocatalytic process. The data show that the full recovery of enzyme activity coincides with covalent FAD incorporation. It also corroborates the previously observed effect of covalent FAD binding: the covalent linkage increases the activity of VAO by one order of magnitude [15]. The observed initial rapid increase in enzyme activity upon incubating with FAD is likely caused by noncovalent binding of the FAD cofactor.

To investigate the effect of FAD concentration on the rate of covalent flavinylation, 1.1 μM apo VAO was incubated with various FAD concentrations (range = 1–500 μM). The rate of flavinylation was measured by monitoring the increase in enzyme activity during the incubation with FAD. As shown in Fig. 6B, the rate of flavinylation exhibits a hyperbolic

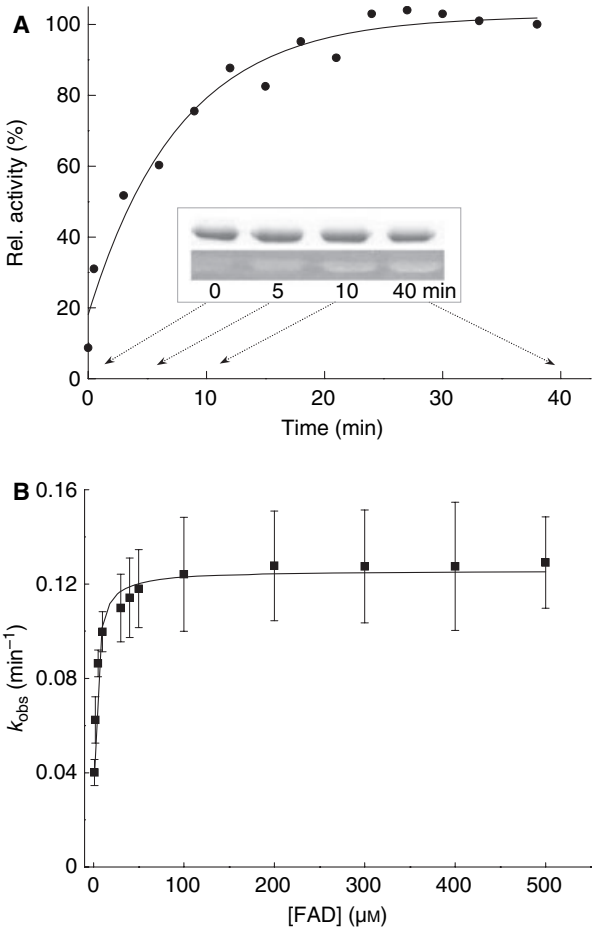


Fig. 6. (A) Enzyme activation and FAD incorporation of apo VAO. The reaction was initiated by adding apo VAO (1.1 μM) to 50 mM potassium phosphate buffer (pH 7.5) containing 500 μM FAD at 25 °C. Aliquots were withdrawn at the indicated times and assayed for VAO activity. The solid line is a fit of the data to a single-exponential equation, $A = A_0 + \Delta A(1 - e^{-kt})$, where A is the observed activity, A_0 is the activity at time zero, ΔA is the total increase in activity and k is the apparent first-order rate constant. The inset shows the time-dependent protein staining and fluorescence intensity upon SDS/PAGE of selected samples. (B) FAD dependence of the rate of enzyme activation of apo VAO. The observed rate of enzyme activity recovery was measured (Fig. 5A) in the presence of 1–500 μM FAD.

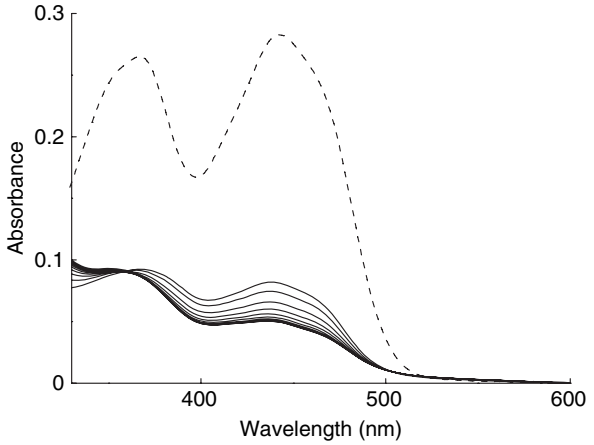


Fig. 7. Spectral analysis of anaerobic FAD incorporation in apo VAO. Spectra were recorded at 0.5, 2, 4, 7, 10, 15, 20, 25 and 30 min, respectively, after mixing 150 μM apo VAO with 100 μM FAD in 50 mM potassium phosphate buffer (pH 7.5) containing 100 mM glucose and glucose oxidase (10 U·mL^{-1}) at 25 °C. The spectrum obtained upon opening cuvette to air is also shown (- - -).

dependence with respect to the concentration of FAD. The maximum rate and the K_{FAD} for the covalent flavinylation reaction were estimated to be 0.13 ± 0.02 min^{-1} and 2.3 ± 0.2 μM, respectively. Because covalent flavinylation is predicted to result in hydrogen peroxide formation, the rate of hydrogen peroxide formation upon incubating apo VAO with 500 μM FAD was measured. The observed rate of hydrogen peroxide formation (k = 0.13 ± 0.01 min^{-1}) is in good agreement with the rate of activity recovery under similar conditions (500 μM FAD, 50 mM potassium phosphate).

Covalent flavinylation may involve the formation of a reduced flavin intermediate [6]. For direct proof of the formation of a reduced FAD enzyme intermediate, the FAD incorporation in apo VAO was monitored by collecting UV-visible spectra upon mixing apo enzyme with FAD under anaerobic conditions (Fig. 7). The anaerobic reaction of 150 μM apo VAO with 100 μM FAD resulted in a time-dependent reduction of the flavin, which took 30 min to complete. On the basis of the extinction coefficients of VAO-bound oxidized FAD (12.5 mM^{-1}·cm^{-1}) and VAO-bound reduced FAD (2.2 mM^{-1}·cm^{-1}), a complete reduction of FAD was observed. Immediate and complete reoxidation of the reduced flavin was observed upon aeration of the sample. This indicates that reoxidation of the reduced covalently linked FAD is not limiting the rate of cofactor coupling. The reoxidation of the reduced flavin intermediate has been suggested to occur by a reduction of molecular oxygen to hydrogen peroxide. Upon mixing 3.56 μM apo VAO with 500 μM FAD, horseradish peroxidase and chromogenic peroxidase substrates, an almost equimolar amount of hydrogen peroxide (3.46 μM) could be detected.

Discussion

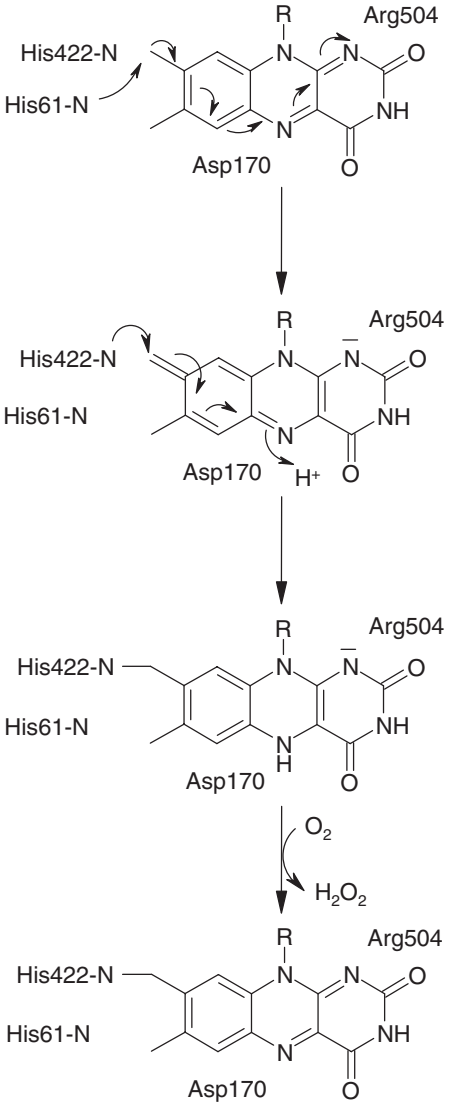
In the present study, apo wild-type VAO was produced using a riboflavin-dependent *E. coli* strain as the heterologous expression host. The apo VAO thus obtained

could be reconstituted with FAD, resulting in full recovery of activity and concomitant full covalent incorporation of the FAD cofactor. Other flavins (i.e. FMN and riboflavin) were not covalently incorporated. Apo VAO displayed a similar affinity for FAD and ADP, indicating that the ADP moiety of FAD plays an essential role in cofactor recognition. The affinities for ADP and FAD binding were similar to those measured with the H61T VAO mutant, which is incapable of forming a His422-FAD bond [16]. The crystal structures of H61T in its apo, ADP-bound and FAD-bound forms have revealed that apo VAO is able to form a preorganized active site and cofactor binding cavity. Binding of ADP or FAD does not induce significant structural changes of the cofactor binding cavity or active site residues. Nevertheless, subtle changes in conformation and/or flexibility in other parts of the protein may explain the differences observed with respect to stabilization of oligomeric states.

MS analyses and the tryptophan fluorescence titration experiments revealed that the binding of FAD to apo VAO is a fast process. However, formation of the covalent flavin–protein bond is much slower ($0.13\ min^{-1}$), as shown by tandem MS analysis and SDS/PAGE. The MS analysis of holo formation of VAO also indicated that noncovalent binding of FAD shifts the monomer/dimer/octamer equilibrium towards the dimeric and octameric species. Such an effect on oligomerization has also been observed during the holo formation of apo H61T VAO [25].

The covalent incorporation of FAD results in a higher enzyme activity. This is in agreement with a previous study demonstrating that the covalent FAD–protein bond increases the redox potential of the cofactor, thereby increasing enzyme activity [15]. The observed rate of covalent flavinylation and enzyme activation exhibits a hyperbolic dependence on the concentration of FAD, indicating that the covalent flavinylation reaction is preceded by enzyme–FAD complex formation. For apo 6-hydroxy-D-nicotine oxidase, the rate of flavinylation also exhibits saturation kinetics with respect to FAD [28]. By contrast, in a study of covalent flavinylation of monomeric sarcosine oxidase, FAD dependent reconstitution kinetic behavior was reported to show an apparent linear dependence on the FAD concentration [6]. The kinetic data for VAO indicate that covalent incorporation involves the formation of a tight FAD–protein complex ($K_{d,\ FAD} = 0.7 \pm 0.2\ \mu M$), which subsequently (auto)catalyzed the formation of a covalent FAD–protein bond.

Evidence for the occurrence of a reduced flavin intermediate in the autocatalytic covalent flavinylation reaction was obtained by anaerobic mixing of apo VAO with FAD, which yielded fully reduced FAD. The reduced flavin was readily reoxidized by molecular oxygen because a stoichiometric amount of hydrogen peroxide was formed upon covalent coupling of FAD. Based on the results described above and the previous studies on VAO, we propose an autocatalytic covalent flavinylation mechanism that is similar to that described for 6-hydroxy-D-nicotine oxidase (Scheme 1) [15,16,29]. All residues that are predicted to be directly involved in covalent flavinylation (His61, Asp170, His422 and Arg504) have been mutated in previous studies. Replacing these residues yielded proteins with no or limited covalent FAD incorporation. The final



Scheme 1. Postulated mechanism for autocatalytic covalent flavinylation of VAO. All active site residues that were demonstrated to affect the covalent flavinylation of VAO upon replacement are shown [15,16,29].

step of covalent flavinylation involves the transfer of two electrons. Although many flavoproteins are unable to utilize molecular oxygen as an electron acceptor, it is not surprising that VAO, being an oxidase, is able to do so. Nevertheless, we cannot rule out that other electron acceptors may facilitate the covalent flavinylation reaction *in vivo*. Intriguingly, we have found that anaerobically grown *E. coli* cells express VAO in a fully covalently flavinylated form (data not shown), suggesting that another electron acceptor can promote covalent flavinylation.

In the present study, the covalent flavinylation process was thoroughly investigated by MS and other techniques. The data obtained reveal that the covalent flavinylation of apo VAO is a relatively slow and autocatalytic process. The data show that the formation of the covalent FAD–protein bond does not play a role in stabilization of oligomeric VAO forms. This is in line with the hypothesis that the primary rationale behind the post-translational autocatalytic covalent flavinylation of VAO lies in increasing the oxidative power of the oxidase by increasing the redox potential [15].

Experimental procedures

Chemicals

Restriction enzymes, DNA polymerase and T4 DNA ligase were obtained from Roche (Basel, Switzerland). Horseradish peroxidase was purchased from Sigma-Aldrich (St Louis, MO, USA). Vanillyl alcohol (4-hydroxy-3-methoxybenzyl alcohol), 4-aminoantipyridine and 3,5-dichloro-2-hydroxybenzenesulfonic acid were purchased from Acros (Geel, Belgium). DNA samples were purified using the QIAquick gel and purification kit from Qiagen (Hilden, Germany). *E. coli* TOP10 competent cells and the pBAD/myc-HisA vector were purchased from Invitrogen (Carlsbad, CA, USA). *E. coli* BSV11 (CGSC#6991) was obtained from the Coli Genetic Stock Center (MCDB Department, Yale University, CT, USA).

Expression and purification of apo VAO

The *vao* gene was amplified using plasmid pVAO [24] as the template, 5′-CACCATATGTCCAAGACACAGGAA TTC-3′ as the forward primer (*Nde*I site is underlined) and 5′-CACAAGCTTTTACAGTTTCCAAGTAACATG-3′ as the reverse primer (*Hin*dIII site is underlined). After amplification, the DNA was digested with *Nde*I and *Hin*dIII, purified from agarose gel, and ligated between the same restriction sites in pBADNk, a pBAD/myc-HisA-derived expression vector in which the original *Nde*I site is removed and the *Nco*I site is replaced by an *Nde*I site. The resulting construct (pBADVAO) was transformed to *E. coli* TOP10 for expression of holo VAO. For expression of apo VAO, the plasmid pBADVAO was transformed to *E. coli* BSV11. *E. coli* BSV11 cells were grown at 37 °C in LB containing riboflavin (50 $\mu g \cdot mL^{-1}$) and kanamycin (100 $\mu g \cdot mL^{-1}$) until D_{600} of 0.5 was reached. The cells were then harvested by centrifugation and washed twice with riboflavin-free LB. The washed cells were resuspended in riboflavin-free LB and grown at 17 °C. L-Arabinose was added to the medium to a final concentration of 0.2% to induce the expression of VAO. Cells were harvested after 3 days. Apo VAO was purified as previously described for holo VAO [12]. The apo VAO concentration was determined using a molar absorption coefficient of 140 $mM^{-1} \cdot cm^{-1}$ at 280 nm [25].

Analytical methods

Enzyme activity was routinely assayed by monitoring the changes in absorption. Activity with vanillyl alcohol and vanillylamine was determined by measuring the formation of vanillin at 340 nm (ε = 14.0 $mM^{-1} \cdot cm^{-1}$ at pH 7.5).

The cofactor incorporation reactions were conducted at 25 °C in 50 mM potassium phosphate buffer (pH 7.5) containing 1–3 μM apo VAO and excess FAD. Reactions were initiated by addition of apo VAO. The kinetics of the incorporation reaction with FAD was monitored by withdrawing small aliquots at various times for VAO activity assays and SDS/PAGE.

The cofactor incorporation reaction was also monitored by measuring the extent of hydrogen peroxide formation during reconstitution of apo VAO with FAD. The reactions were carried out as described above with 20 $U \cdot mL^{-1}$ horseradish peroxidase, and 0.1 mM 4-aminoantipyridine and 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid were added to the reaction mixture to detect the formation of hydrogen peroxide. Formation of the holo enzyme was initiated by the addition of apo VAO. Hydrogen peroxide formation was monitored at 515 nm (ε_{515} = 26 $mM^{-1} \cdot cm^{-1}$) [30]. For anaerobic cofactor incorporation experiments, the enzyme solution (in 50 mM potassium phosphate buffer, pH 7.5) was made anaerobic by adding glucose (100 mM) and glucose oxidase (10 units$\cdot mL^{-1}$) to remove oxygen and flushing with oxygen-free argon in a cuvette (0.2 cm) with a rubber cap. The spectra were recorded after the argon-flushed FAD solution was injected into the enzyme solution with a syringe.

Fluorescence studies

Fluorescence titration experiments were performed essentially as described previously (25). Protein tryptophan fluorescence emission spectra were recorded in the range 310–560 nm. The excitation/emission wavelengths were set at 295/340 nm. The protein emission fluorescence was recorded in the presence of various concentrations of FAD and ADP in 50 mM potassium phosphate buffer

(pH 7.5). After addition of FAD or ADP, the sample was incubated for 4 min in the dark before measuring the emission intensity.

MS

For nanoflow ESI-MS experiments, enzyme samples were prepared in aqueous 50 mM ammonium acetate buffer (pH 6.8). The holoenzyme formation was initiated by addition of FAD using a four-fold molar excess. VAO samples (4 μM) were introduced into a modified quadrupole Q-TOF 1 mass spectrometer (Micromass, Manchester, UK), operating in positive ion mode, by using gold-coated needles. The needles were made from borosilicate glass capillaries (Kwik-Fil; World Precision Instruments, Sarasota, FL, USA) on a P-97 puller (Sutter Instruments, Novato, CA, USA) and coated with a thin gold layer by using an Edwards Scancoat six Pirani 501 sputter coater (Edwards Laboratories, Milpitas, CA, USA). Native mass spectra were acquired on a modified Q-TOF 1 instrument under conditions optimized for the transmission of noncovalent complexes [31,32]. The needle and sample cone voltage were 1350 V and 160 V, respectively, and the collision energy was 50 V.

For tandem MS, ions were isolated in the quadrupole analyzer and accelerated into an argon-filled linear hexapole collision cell. The capillary voltage was typically set at 1350 V, the cone voltage at 160 V, and the collision energy was 200 V. The pressure in the first vacuum stage of the instrument was increased by reducing the pumping efficiency of the rotary pump to 10 mbar. In the second hexapole chamber, the pressure was 4×10^{-3} mbar; the third vacuum chamber, containing the quadrupole, had a pressure of 6.7×10^{-4} mbar. Pressure conditions in the collision cell were 1.5×10^{-2} mbar and 2×10^{-6} mbar in the TOF chamber, with argon at a pressure of 2×10^{-2} mbar.

For measurements under denaturing conditions, the protein was diluted in a solution containing 50% acetonitrile and 0.2% formic acid and analyzed with the LC-T nanoflow ESI orthogonal TOF mass spectrometer (Micromass, Manchester, UK).

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